Endothelins, pseudo-obstruction and Hirschsprung’s disease

It has been known since the 1950s that the enteric nervous system is formed from cells that arise from the neural crest. The enteric neurons mainly arise from the vagal neural crest of the developing hind brain and colonise the gut in a rostro-caudal migration but some seem to arrive in the hind gut from the lumbosacral level via a caudo-rostral wave of colonisation. The neural crest cells that migrate and colonise the gut are committed to become neuroblasts or neuronal support cells, glioblasts; however, differentiation into neurones and glial cells seems not to take place until they have reached their final resting places in the gut. Movement through the gut mesenchyme, survival in the gut and differentiation into mature cells is strongly influenced by contacts with the microenvironment which consists of other cells in the mesenchyme, neural crest, and the extracellular matrix. The extracellular matrix components provide directional clues to migrating neural crest cells and together with neighbouring cells provide some of the signals for crest cell differentiation. For example, the appearance of neural crest cells in the gut is preceded by expression of extracellular matrix molecules and other factors such as glial derived neurotropic factor (GDNF) ensure survival of committed neuroblasts. Thus defects of the neural crest cells themselves or alteration of the micro-environment of the migratory pathway may result in defects of development of the enteric nervous system. In humans this disordered development results in the most commonly presenting forms of chronic idiopathic intestinal pseudo-obstruction, congenital enteric neuromuscular disease. In Hirschsprung’s disease’s defects in at least two different cell signalling systems, ret/GDNF and endothelin-3/endothelin B receptor, cause the aganglionicosis.

The endothelin system’s important role in the development of the enteric nervous system has become apparent in the past four years or so when mice with targeted disruption of endothelin B receptor (ETR-B) and endothelin-3 (ET-3) were found to have congenital distal intestinal aganglionicosis. The endothelins are a family of three peptides, endothelin-1, -2 and -3, coded for by distinct but related genes and act on cells via two G-protein coupled receptors ETR-A and ETR-B. The endothelins are synthesised as much larger proproteins which are cleaved by an endothelin converting enzyme (ECE-1) to produce the active 21 amino acid peptide. Of the three endothelins it is ET-3 which is so important in the enteric nervous system and binding of ET-3 to ETR-B on vagal neural crest cells is required for colonisation of the hind gut.

Mutations of either ETR-B or ET-3 have been identified in several naturally occurring animal models of Hirschsprung’s disease, the piebald lethal mouse and the lethal spotted mouse respectively. The ovoar-ovale white foal also has a significant mutation of ETR-B with a single amino acid substitution in the first transmembrane spanning domain of the ETR-B gene. The lethal spotted mouse carries a mutation in its ET-3 gene which prevents proteolytic activation of the peptide. Mutation analysis of children with Hirschsprung’s disease has shown that about 10% carry mutations of either ETR-B, ET-3 or ECE-1. The effects of these genetic defects is to curtail neural crest migration in the distal colon and this is associated with localised overexpression of extracellular matrix molecules. Using transgenic lines of mice which are either ETR-B deficient or ET-3 deficient, Kapur and colleagues have shown that in ETR-B deficient mice enteric nervous system precursors can colonise the murine hind gut when they are surrounded by wild type enteric nervous system precursors. Further wild type enteric nervous system precursors will fail to colonise the hind gut when surrounded by ETR-B deficient ones. This strongly suggests that the enteric nervous system precursors signal ETR-B activation to those nearby and that when this signal is of sufficient intensity an ETR-B deficient crest cell can develop normally. It is thus clear that the interaction between the migrating neural crest cells and the mesenchymal environment of the hind gut is of critical importance in achieving normal innervation of the colon. The mechanism of the terminal aganglionicosis that occurs either in the absence of ET-3 or ETR-B however remains unclear.

Despite the increasing understanding of the role of endothelins in the developing enteric nervous system, little work has been done in normal mice or men regarding the timetable of activity or the spatial orientation of these molecules in the developing embryonal gut. On page 246 of this issue Leibl et al describe the temporal and spatial expression of ET-3 and ETR-B in CD1 mouse embryos. They show clearly that ETR-B is confined to migrating neural crest cells and ET-3 to mesenchymal cells initially of the caecum but with a gradient extending rostrally into the small intestine and caudally into the proximal colon. Interestingly by 14 days postcoitum the ETR-B mRNA signal in the colon was stronger than in the more proximal part of the gut at this or earlier stages, perhaps, suggesting that ETR-B is expressed by both vagal and sacral neural crest cells.

The present results add to the growing body of work emphasising the importance of the gut mesenchyme in determining regional identity along the gut primordium and also in the regulation of region specific innervation of the gastrointestinal tract. The mechanisms that regulate expression of ET-3 and ETR-B genes are currently unknown. It is clear however that the rostro-caudal specification of the gastrointestinal tract is likely to involve a spatial, temporal and combinatorial patterns of expression of homeobox genes, the so called enteric hox code. In chick embryos there is clearly overlapping expression of the genes Hox A-9, -10 and -11 and we have recently produced some preliminary data demonstrating specific spatial, temporal and combinatorial expression patterns of hox genes A4, B4, D4, A5 and C5 in developing murine gut. The relation between caecum specific hox gene expression and ET3 and ETB-R is currently unknown but they are certainly candidate downstream molecules for these developmental control genes. A number of transgenic animal models provide evidence of the importance of homeobox genes in the control of morphogenesis of the gut and these include the “knock out” of ENX, causing increased innervation of the hind gut, and over expression of hox A4, resulting in megacolon. Thus this family of genes and their
downstream targets are of importance within the genetic hierarchy of gut morphogenesis. Delineation of the genes comprising the enteric box code, their downstream targets and their spatiotemporal patterns of expression is an essential and integral part of understanding the molecular events underlying the devastating diseases which cause pseudo-obstruction and Hirschsprung’s disease in humans. Such knowledge may enable antenatal diagnosis in some families and will be essential for the development of neuronal transplant strategies for the treatment of enteric neuropathic diseases.

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Back to the whale bone?

Most doctors with any practical experience of achalasia would be willing to admit that the disorder often provides considerable professional satisfaction. Firstly, it can be very satisfying to make the diagnosis. Far too often, patients will have suffered from gradually worsening dysphagia for many years and the diagnosis will have been missed at earlier consultations. The second moment of satisfaction can be enjoyed when the symptoms are relieved immediately after a procedure is performed. Secondly, it can be very effective than surgery and dilatation,⁴ and is therefore used mainly for short periods—for example, while the patient is on a waiting list for a more definitive procedure.

Since 1994, a fourth option has emerged, namely intra-sphincteric injection of botulinum toxin type A, a toxin produced by Clostridium botulinum, which inhibits acetylcholine release from nerve endings.⁵ This approach was shown to lead to short term symptom relief in up to 90% of patients.⁶ By six months, 20 (65%) of the 31 patients treated were still in remission. The endoscopic injection procedure is simple and no major complications have been reported as yet. A question that remains unanswered is how botulinum toxin injection compares with endoscopic myotomy. In the early studies with botulinum toxin concerns the duration of its effect in comparison with surgery and pneumatic dilatation.

In this issue (see page 231) Vaezi et al describe a randomised study in which they compared the immediate and long term efficacy of botulinum toxin with pneumatic dilatation. Their study is the first that formally compares these two treatment modalities in a prospective way. The most important result of the study, from a clinical point of view, is that botulinum toxin injection resulted in a significantly lower remission rate at 12 months than did pneumatic dilatation (32% compared with 70% of patients in complete remission). As an explanation for the apparent difference between the outcome of their study and those of some previous reports, the authors rightly highlight the fact that in previous studies of botulinum toxin in achalasia, repeat injections were given to patients who relapsed shortly after the initial injection. In an earlier study that compared botulinum toxin injection with pneumatic dilatation, only patients who did not respond to botulinum toxin treatment were pneumodilated, rendering the comparison unfair.⁷

Vaezi and colleagues also examined the response of a number of objective parameters to treatment with botulinum toxin and pneumatic dilatation. Changes in diameter and length of the barium column at radiographical examination of the oesophagus paralleled changes in symptom scores. Somewhat surprisingly, however, botulinum toxin, in contrast to pneumatic dilatation, did not have a statistically significant effect on LOS pressure. Even at one month after botulinum injection no reduction in LOS pressure was found. This finding is in contrast with observations made in earlier studies. The possibility that
measurement of mid-expiratory rather than of end-
expiratory LOS pressure might have obscured an effect of 
botulinum toxin is discarded in the discussion section of 
the paper, although the authors do not provide us with the 
measurements.

The results of the study by Vaezi and colleagues raise 
more doubts on the clinical value of botulinum toxin treat-
ment in achalasia than hitherto expressed. It is important 
to those actively involved in the treatment of patients with 
achalasia to consider the results of Vaezi et al’s study care-
fully. As the aim of treatment in achalasia, a life-long 
disease, is to reduce symptoms with a minimum number of 
interventions during the patient’s lifetime, a new treatment 
that has to be repeated frequently is likely to be less satis-
factory, both to the patient and to the doctor, than the 
existing range of potential treatments.

As has been the case with many other new therapeutic 
options for various other diseases, the initial enthusiasm for 
botulinum toxin treatment in achalasia may have been too 
great. It seems that the pendulum is swinging back again. It 
is highly unlikely, though, that it will swing back to the 
whole bone approach!

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injections versus placebo and pneumatic dilation in achalasia. Gastroenterol-

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Genes means pancreatitis

Identifying the molecular mechanisms responsible for 
acute and chronic pancreatitis in humans is one of the most 
difficult problems in modern science. Major obstacles 
include the inaccessibility of the human pancreas to obser-
vation, the unpredictability of disease onset, the non-
specific nature of abdominal pain early in the course of 
acute pancreatitis, an inability to biopsy the pancreas 
safely, difficulty in distinguishing initiating events from 
the concomitant inflammatory response, and the obvious 
problems of investigating a tissue that self-destructs during 
the disease process. Even fundamental questions as to 
whether pancreatitis begins in the acinar cell or through 
pathology related to the pancreatic ducts continue to be 
debated.1,2 Animal models also fail to provide critical 
insights, partly because of the artificial methods used to 
induce pancreatitis.3,4

The discovery of the mutations in the cationic trypsin-
gen gene responsible for hereditary forms of pancreatitis in 
American and European kindreds5–7 provided tremendous 
insights into the mechanism of acute and chronic pancrea-
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trypsinogen R117H mutation eliminates a key hydrolysis 
site on the chain connecting the two globular domains of 
trypsin that is part of a fail-safe trypsin inactivation mech-
anism. Rather than being autolysed, prematurely activated 
mature trypsin remains active within the pancreas, 
activates all other digestive enzymes, leads to acinar cell 
autodigestion and, therefore, acute pancreatitis. The 
second major insight was that the chronic pancreatitis 
commonly seen in patients was associated with mutations 
in trypsinogen. This observation suggests that recurrent 
acute pancreatitis may lead to chronic pancreatitis.2,6,7

Families with the cationic trypsinogen R117H and N21I 
mutations have now been identified in Caucasians throughout the United States and Europe.

In this issue, Nishimori et al (see page 259) report the 
presence of the same two cationic trypsinogen gene muta-
tions in Japanese kindreds with hereditary pancreatitis as 
seen in Caucasians. Additional polymorphisms in the cati-
onic trypsinogen gene were also reported, but they either 
fail to result in an amino acid substitution or segregate with 

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(See article on page 259)

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tions in Japanese kindreds with hereditary pancreatitis as 
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fail to result in an amino acid substitution or segregate with 
the pancreatitis phenotype. Thus, this report expands the 
observation of pancreatitis causing cationic trypsinogen 
mutations to Asians and further defines the limits of pan-
creatitis causing mutations to cationic trypsinogen R117H 
and N21I. As hereditary pancreatitis is an autosomal 
dominant disorder, mutations that cause loss of function 
would not cause the syndrome. Furthermore, as hereditary 
pancreatitis is relatively rare and a number families have 
been investigated, it is unlikely that many additional gain-
of-function mutations, such as the R117H mutation, will 
be identified.

The question of why the cationic trypsinogen N21I 
mutation predisposes individuals to pancreatitis was also 
tackled. Computer analysis of the N21I substitution 
suggests that the mutation changes the secondary structure 
in the region of the N21I mutation from a “turn” into a 
“sheet” conformation. The implications of the predicted 
secondary structural changes on the tertiary structure and 
trypsin biology may be important, but remain speculative. 
Other hypotheses on the role of the N21I mutation have 
also been offered8 and could be consistent with these 
predictions. However, proving the actual structural changes 
caused by the mutation and determination of the 
mechanism through which the function of trypsin is altered 
will require further work.

Identification of the same two mutations in the cationic 
trypsinogen gene in kindreds with hereditary pancreatitis of 
both Caucasian and Asian ancestry, combined with the 
finding that potent trypsin inhibitors prevent pancreatitis 
associated with endoscopic retrograde cholangiopancreato-
graphy in humans,6 provides us with strong evidence that 
cationic trypsinogen plays an important role in human acute 
pancreatitis. This represents a major conceptual break-
through. Now, attention can be focused on experimental 
models of acute pancreatitis with premature trypsinogen 
activation, on mechanisms of premature trypsinogen activa-
tion and trypsin stabilisation, and on strategies to limit these 
processes in susceptible individuals.

Another interesting note is that the only mutations iden-
tified to date in patients with hereditary pancreatitis are in 
the human cationic trypsinogen gene. No pancreatitis 
associated mutations have been identified in anionic 
trypsinogen, nor in any of the other digestive enzymes. 
Indeed, human cationic trypsinogen is relatively unique 
among members of the trypsin family in its ability to
Cross-reacting antibodies in coeliac disease?

Most patients with coeliac disease have antibodies to wheat gliadin, reticulin, and endomysium. In 1997 a seminal paper showed that at least a substantial fraction of anti-endomysial antibodies (EMA) recognises the endogenous enzyme tissue transglutaminase (tTG). However, antibodies recognising other antigens can also be found and in this issue (see page 168) Krupicková and colleagues attempt to characterise these antibodies.

Anti-gliadin antibodies (AGA) were isolated from coeliac serum samples using (semi)purified α-gliadin as the substrate. These antibodies were tested for specificity using a synthetic α-gliadin peptide competition assay. This important study is the first of its kind. An interesting finding is that the antibody responses are directed towards a limited set of epitopes. These epitopes do not overlap with peptides recognised by small intestinal HLA-DQ restricted T cells, but our current knowledge is too limited to judge whether this is important. One of the epitopes was the VLPVQQQF peptide, which corresponds to α-gliadin residues 22–30. The glutamains were important, as substitution with glutamic acid removed the inhibitory function of VLPVQQQF. Conversion of Qs to Es (deamidation) by tTG has been implicated recently in the pathogenesis of coeliac disease. However, tTG acts on T cell epitopes of gliadin by specific deamidation of only some of the glutamains and it probably would have been more realistic if only one or some of the Qs in the 22–30 peptide had been replaced.

Although recent studies have focused on coeliac antibodies recognising tTG, other antibody specificities have been reported. Mäki et al described several antigens recognised by EMA but which were not recognised by AGA. Börner et al isolated various components from different animal tissues using serum from patients with coeliac disease. It is unlikely that the antigens reported in these two studies are tTG. As mentioned by Krupicková and colleagues, antibodies cross-reacting with gliadin, enterocytes and calreticulin can also be found. Interestingly, these authors have shown that some of the same peptides that interfere with AGA binding to α-gliadin also affect binding to enterocytes and calreticulin. By doing a sequence similarity search they identified corresponding sequences in α-gliadin and calreticulin. However, it would have been reassuring to see whether synthetic calreticulin peptides could also inhibit binding of AGA to α-gliadin, and further details of the calreticulin preparation used are essential. As with any new and unexpected finding it would also be good to see the cross-reactivity between gliadin and calreticulin reproduced by other investigators.

Can one now conclude that coeliac disease is an autoimmune condition directed against enterocytes and calreticulin? There are some difficulties in accepting such a hypothesis. Calreticulin is an abundant Ca²⁺ binding protein which is expressed in every cell in higher organisms. It has a retrieval signal for endoplasmic reticulum (ER) and the ER is considered to be the major cellular site of localisation. Small amounts of calreticulin may also be present at extra-ER sites including the cell surface. At any rate, an immunological cross-reaction would presumably manifest itself in different organs. Coeliac disease is seen more frequently in IgA deficient individuals, so at least IgA antibodies are not necessary for the disease. If the cross-reactivities were attributable to IgG antibodies, they could give rise to the complement activation known to be present in coeliac lesions. We know that IgG-AGA are not specific as they can be found both in healthy subjects and those with coeliac disease. However, this may not necessarily reflect what is going on in the small intestine. Conversely, cross-reactive AGA could have an effect during the first phases of disease pathogenesis directly following α-gliadin challenge, where the observed phenomenon might fit with a rapid antibody recognition event. Whether cross-reactive antibodies recognising enterocytes are capable of inducing apoptosis is still an open question.

Finally, a comment can be made on the usage of the “molecular mimicry” model as an explanation for the putative autoimmune component in coeliac disease. As Michael Bevan defined it, molecular mimicry is important for the induction phase of autoimmunity, where an infectious agent triggers an autoimmune loop, which persists even after the infection has been cleared. The complete remission seen in almost all coeliac patients after...
withdrawal of cereal proteins from the diet is difficult to reconcile with this concept.

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